Identification and Functional Characterization of Zebrafish Solute Carrier Slc16a2 (Mct8) as a Thyroid Hormone Membrane Transporter

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Most components of the thyroid system in bony fish have been described and characterized, with the notable exception of thyroid hormone membrane transporters. We have cloned, sequenced, and expressed the zebrafish solute carrier *Slc16a2* (also named monocarboxylate transporter *Mct8*) cDNA and established its role as a thyroid hormone transport protein. The cloned cDNA shares 56–57% homology with its mammalian orthologs. The 526-amino-acid sequence contains 12 predicted transmembrane domains. An intracellular N-terminal PEST domain, thought to be involved in proteolytic processing of the protein, is present in the zebrafish sequence. Measured at initial rate and at the body/rearing temperature of zebrafish (26 C), T_3 uptake by zebrafish Slc16a2 is a saturable process with a calculated Michaelis-Menten constant of 0.8 μM T_3. The rate of T_3 uptake is temperature dependent and Na^+ independent. Interestingly, at 26 C, zebrafish Slc16a2 does not transport T_4. This implies that at a normal body temperature in zebrafish, Slc16a2 protein is predominantly involved in T_3 uptake. When measured at 37 C, zebrafish Slc16a2 transports T_4 in a Na^+-independent manner. In adult zebrafish, the *Slc16a2* gene is highly expressed in brain, gills, pancreas, liver, pituitary, heart, kidney, and gut. Beginning from the midblastula stage, *Slc16a2* is also expressed during zebrafish early development, the highest expression levels occurring 48 h after fertilization. This is the first direct evidence for thyroid hormone membrane transporters in fish. We suggest that Slc16a2 plays a key role in the local availability of T_3 in adult tissues as well as during the completion of morphogenesis of primary organ systems. **(*Endocrinology* 152: 5065–5073, 2011)**

Thyroid hormones regulate many important functions in bony fish (Osteichthyes) such as growth, skin pigmentation, morphogenesis during early development, and metamorphosis (1). The main secretion product of the thyroid gland is thyroxine or 3,5,3′,5′-tetraiodothyronine (T_4). In extrathyroidal tissues, T_4 is deiodinated by intracellular deiodinases to obtain T_3, the primary effect of which is on the transcriptional regulation of target genes. After entering the cell and/or after T_4 deiodination, T_3 binds to thyroid hormone receptors that translocate to the nucleus. The formation of T_3-receptor complexes is a critical step in the positive or negative regulation of target gene transcription rates and the subsequent regulation of protein synthesis. A key requirement of this mechanism of action is that thyroid hormones gain access to the cytoplasm by crossing the plasma membrane barrier.

Thyroid hormones are lipophilic, and for long it has been assumed that they traverse biological membranes by means of simple passive diffusion. However, it is now firmly established that thyroid hormone membrane transport is mediated by specific intrinsic membrane proteins (2). Early studies in fish hepatocyte plasma membranes

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-1166 Received April 28, 2011. Accepted September 9, 2011.
First Published Online September 27, 2011

Abbreviations: cds, Coding sequence; hpf, hours post-fertilization; RT-qPCR, real-time quantitative PCR; SLC, solute carrier; TMD, transmembrane domain.

have shown that passive diffusion represents less than 10% of the total thyroid hormone transport (3, 4), clearly indicating an important role for transporter-mediated pathways. Unfortunately, nothing is known as yet about piscine thyroid hormone transporters.

Several thyroid hormone membrane transporters have been identified in mammals (5, 6), and all of them belong to the solute carrier (SLC) superfamily, which is subdivided into 47 families (7). To date, SLCO1C1/Slco1c1 (8–10), SLC16A2/Slc16a2 (11–13), and SLC16A10/Slc16a10[1] (14) have been shown to transport thyroid hormone with relatively high affinities. Of these SLC proteins, rat Slc16a2 and human SLC16A2 (also known as monocarboxylate transporter MCT8/Mct8) have been identified as specific thyroid hormone membrane transporters (11, 14). The key role of SLC16A2 in thyroid hormone signaling is dramatically demonstrated by the severe psychomotor retardation and neurological abnormalities in young patients with dysfunctional mutations in the *SLC16A2* gene (2, 15).

Orthologs of mammalian thyroid hormone membrane transporters have also been described in nonmammalian species such as *Xenopus tropicalis*, where putative transporters are suggested to play a key role in metamorphic events (16). In birds, the functional characterization of quail Slco1c1 (also known as organic anion transporting polypeptide Oatp1c1) revealed that this membrane protein is a highly specific transporter for T_4 but not for rT_3, which latter substrate is transported by its mammalian counterpart (17).

The zebrafish (*Danio rerio*) is a member of the cyprinid family and a widely used model species for other vertebrates. Many of the components of the zebrafish thyroid axis have been characterized (18, 19). However, no thyroid hormone membrane transporters have been identified and characterized to date. The Slc16 family is present in zebrafish (20) and could well contain Slc16a2, which would be the piscine ortholog of the mammalian specific thyroid hormone transporter SLC16A2/Slc16a2. The aims of the present study were 1) to identify the zebrafish ortholog of the mammalian SLC16A2/Slc16a2 in the zebrafish genome, 2) to investigate the pattern of *Slc16a2* gene expression in zebrafish tissues and during early development, and most importantly, 3) to establish whether zebrafish Slc16a2 protein transports thyroid hormone.

[1] Protein:*gene* nomenclature: human, SLCO1C1:*SLCO1C1*, SLC16A2:*SLC16A2*, SLC16A10:*SLC16A10*; other animals, Slco1c1:*Slco1c1*, Slc16a2:*Slc16a2*, Slc16a10:*Slc16a10*.

Materials and Methods

Fish and sampling procedures

Adult wild-type zebrafish (1 yr of age) were commercially obtained (De Maanvis, Nijmegen, The Netherlands) and reared in 2-liter tanks at 26 C with recirculating, UV light-treated Nijmegen city tap water. Fish were fed daily with Tetra-min fish feed (Tetra, Melle, Germany) at a ration of 2.5% of the total body weight. Sampling occurred 1 h after feeding; fish were anesthetized in 0.1% (vol/vol) 2-phenoxyethanol (Sigma Chemical Co., St. Louis, MO) and killed by spinal transection. Organs were collected in sterilized 1.5-ml Eppendorf vials, immediately frozen on dry ice, and stored at −80 C until further analyses (tissue distribution and cloning). For the analysis of the temporal pattern of *Slc16a2* gene expression during early development, groups of six zebrafish (wild-type) embryos or larvae were collected 3, 6, 12, 24, 48, and 72 h post-fertilization (hpf). The following developmental stages were determined: midblastula (3 hpf), gastrula (6 hpf), 6–10 somite segmentation (12 hpf), pharyngula (24 hpf), late pharyngula (48 hpf), and yolk sac larvae (72 hpf). Animal procedures were performed in accordance with national legislation and were approved by the ethical review committee of Radboud University Nijmegen (The Netherlands).

RNA isolation and cDNA synthesis

RNA was isolated from zebrafish tissues or individual whole embryos using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was precipitated in ethanol, washed, and dissolved in nuclease-free water. RNA concentrations were measured spectrophotometrically, and 1 μg (tissues) or 500 ng (embryos and larvae) of total RNA was used for cDNA synthesis. cDNA synthesis was carried out as previously described (21), and samples were stored at −20 C until further use.

Design of gene-specific primers for zebrafish *Slc16a2* cloning

TBLASTN searches were performed on the zebrafish whole-genome shotgun database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the human SLC16A2 as query amino acid sequence (GenBank accession no. NP_006508). Sequences with a high homology with SLC16A2 were selected. Then, contig Zv8_NA2811.6 (GenBank accession no. CAAK05008556) was predicted to encode exon 1, contig Zv8_NA2811.2 (GenBank accession no. CAAK05008552) exon 2, contig Zv8_NA2811.1 (GenBank accession no. CAAK05008550) exon 3, contig Zv8_scaffold1672.31 (GenBank accession no. CAAK05036070) exon 4, contig Zv8_scaffold1672.30 (GenBank accession no. CAAK05036069) exon 5, and contig Zv8_scaffold1672.30 (GenBank accession no. CAAK05036069) exon 6. Based on this analysis, primers dare.Slc16a2.fw and dare.Slc16a2.rv (Table 1) were designed and used to amplify the coding sequence (cds) of the zebrafish *Slc16a2* gene.

Cloning and sequencing of zebrafish *Slc16a2* gene coding sequence cds

Amplification was carried out by PCR with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The cDNA used for the amplification was synthesized from brain total RNA. Thermocycling conditions were programmed as follows: 1 min initial de-

TABLE 1. Primer oligonucleotide sequences used for cloning of zebrafish *Slc16a2* gene (cds) and for RT-qPCR determinations in different tissues and during early development

Primer	Sequence 5′→3′
dare.Slc16a2.fw	**CACC**ATGCACTCGGAAAGCGATGA
dare.Slc16a2.rv	TCATATGTGTGTCTCCATGTC
dare.qSlc16a2.fw	CGTGCTGATGGGTCTTTGC
dare.qSlc16a2.rv	GGGTCCAACCAGCTCGAA

Primer names that include "q" indicate the primers used for RT-qPCR measurements. Note the sequence CACC (in *bold*) at the 5′ end in primer dare.Slc16a2.fw to enable directional cloning in the vector pcDNA 3.1 and to achieve an adequate Kozak consensus sequence for the initiation of translation processes.

naturation at 98 C; 35 cycles of denaturation (98 C, 10 sec), annealing at primer-specific temperatures (58 C, 20 sec), and extension (72 C, 2 min); and a final extension step at 72 C for 10 min. Product size was verified by electrophoresis on a 1% agarose gel. The band predicted to contain zebrafish *Slc16a2* cds was excised, and cDNA was purified using the QIAEX II gel extraction kit (QIAGEN, Hilden, Germany). Subsequently, zebrafish *Slc16a2* cDNA was cloned using the pcDNA 3.1 directional TOPO expression kit (Invitrogen), and the pcDNA 3.1 construct was transformed into competent *Escherichia coli* cells. Plasmid DNA was isolated from positive clones using the Quantum Prep plasmid midiprep kit (Bio-Rad, Hercules, CA) and stored at −20 C until further use. The cDNA inserted (putative zebrafish *Slc16a2* cds) into the pcDNA 3.1 vector was sequenced by the sequencing facility at Universitair Medisch Centrum St. Radboud (Nijmegen, The Netherlands). The sequence obtained was submitted to the DDBJ/EMBL/GenBank databases under accession number FN687448 (cds) and CBK52149 (protein). Transmembrane domains of zebrafish Slc16a2 protein were predicted using hidden Markov models (TMHMM server version 2.0 at http://www.cbs.dtu.dk/services/TMHMM/) (22). PEST domains [regions rich in proline (P), aspartate (D), glutamate (E), serine (S), and threonine (T) and flanked by positively charged residues, which are thought to target proteins for rapid degradation] were predicted using the epestfind algorithm (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind). Hydrophilic stretches flanked by positively charged amino acids [arginine (R), histidine (H), or lysine (K)] of not fewer than 12 amino acids length and containing at least one proline (P), one aspartate (D) or glutamate (E), and at least one serine (S) or threonine (T) were considered valid PEST domains.

Phylogenetic analysis

Multiple sequence alignments were carried out using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) (23). A phylogenetic tree was constructed based on amino acid difference (*p*-distance) with the neighbor-joining algorithm (pairwise deletion) using the MEGA software version 4 (24). The reliability of the tree was assessed by bootstrapping using 1000 replications. Only full-length coding sequences were used for analysis.

Thyroid hormone uptake studies

COS-1 cells were seeded in six-well plates at densities of 2×10^5 cells per well. Per plate, two wells served as blanks and were not seeded with cells. Cells were grown in DMEM/F12 medium supplemented with GlutaMAX (1×; Invitrogen) and containing 9% bovine calf serum (HyClone, Logan, UT) at 37 C. Blank wells were rinsed with the same volume of DMEM/F12 medium as used in the wells containing cells. Forty-eight hours after seeding (at 75% confluence), cells were transfected in duplicate with 1 μg empty pcDNA 3.1 vector (control cells) or vector containing zebrafish *Slc16a2* cDNA using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours after transfection, plates were preincubated at 26 C (the rearing temperature of our zebrafish) or 37 C for 20 min and washed with prewarmed incubation medium (DMEM/F12 plus 0.1% BSA). BSA replaced the bovine calf serum during assays. The reasons for this replacement are two: 1) presence of thyroid hormones in the bovine calf serum and 2) BSA provides a buffer of loosely bound ligand in the unstirred water layer surrounding the cell, from which ligand (thyroid hormones) would be rapidly depleted in the absence of BSA (25). Plates were then incubated at 26 C or 37 C in 1.5 ml incubation medium to which T_3 or T_4 and 2×10^5 cpm ^{125}I-labeled T_3 or T_4, respectively, were added. T_3 and T_4 concentrations as well as incubation times are indicated in the legends to figures. Radiotracer was purified on a 10% (wt/vol) Sephadex LH-20 mini-column shortly before use as described by Mol and Visser (26). After incubation, wells were washed with incubation medium at room temperature and rinsed with 1 ml 0.1 N NaOH to lyse the cells. NaOH fractions were aspirated and counted for ^{125}I radioactivity. Cellular thyroid hormone uptake was calculated as femtomoles thyroid hormone contained in the cell lysates. Results were corrected for the amount of ^{125}I radioactivity associated with the walls of the culture wells by subtracting the amount of ^{125}I radioactivity extracted from the wells incubated without cells (blanks).

To evaluate Na^+ dependence of thyroid hormone transport by zebrafish Slc16a2, cells were incubated in a medium containing 142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES, and 0.1% BSA (pH 7.4) or in a medium where Na^+ was replaced with an equimolar amount of choline$^+$. These media were also used during washing steps. Concentrations of thyroid hormone and incubation conditions are indicated in the legends to figures.

Before experimentation, the required ratio FuGENE 6 to DNA (microliters per microgram) was optimized using the vector pEGFP-C1 (BD Biosciences Clontech, Sparks, MD). A 3:1 ratio resulted in the highest transfection efficiency (*i.e.* 25–30%) and was used during all experiments.

Temporal pattern of zebrafish *Slc16a2* gene expression during early development and tissue distribution in adult tissues

The relative expression of zebrafish *Slc16a2* was assessed by real-time quantitative PCR (RT-qPCR) in adult tissues and embryos/larvae. Primers were designed using the Primer Express software (Applied Biosystems, Carlsbad, CA), and they are shown in Table 1. Five microliters of cDNA and 600 (adult tissues) or 200 nM (embryos and larvae) forward and reverse primers were added to 12.5 μl SYBR Green Master Mix (Applied Biosystems). The total volume was adjusted to 25 μl with deionized H_2O. RT-qPCR (10 min at 95 C, 40 cycles of 15 sec at

95 C and 1 min at 60 C) was carried out using a GeneAmp 7500 sequence detection system (Applied Biosystems).

Statistics

Net T_3 uptake kinetic data were analyzed using a nonweighted nonlinear regression computer program (SigmaPlot for Windows version 11.0; Systat Software, Inc., Chicago, IL) in which the Levenberg-Marquardt algorithm for least-squares estimation of parameters is employed. Results from thyroid hormone uptake studies are presented as the mean ± SD of two to three independent experiments performed in duplicate. In experiments where temperature and Na^+ dependence was assessed, statistical significance was determined using Student's unpaired *t* test and accepted at $P < 0.05$. The effects of the type of tissue and the time after fertilization on the zebrafish *Slc16a2* gene expression in different tissues and during early development, respectively, were analyzed by a one-way ANOVA followed by a *post hoc* Tukey's test. The significance level was set at $P < 0.05$.

Results

Molecular cloning of zebrafish *Slc16a2* cds

The full-length zebrafish *Slc16a2* cDNA contains 1581 nucleotides, encoding a peptide of 526 amino acids and with an estimated molecular mass of 57 kDa (Supplemental Fig. 1). The amino acid identity between zebrafish Slc16a2 protein and its predicted amino acid sequence obtained by analysis of the whole-genome shotgun database is 99%.

Sequence identity with mammalian SLC16A2/Slc16a2 proteins from human, mouse and rat (the only species where these proteins have been demonstrated to transport thyroid hormones) is 57, 57, and 56%, respectively (Fig. 1). In general, the 12 hydrophobic transmembrane domains (TMD) are particularly conserved between zebrafish Slc16a2 protein and its mammalian counterparts. The loops between TMD are less conserved, especially the large intracellular loop between TMD6 and TMD7. Two amino acids, Arg^{445} and Asp^{498}, which are supposed to be critical for the molecular interactions between the transporter and the thyroid hormone substrate in humans (27) are present in zebrafish Slc16a2 protein. An intracellular N-terminal PEST domain [a region rich in proline (P), aspartate (D), glutamate (E), serine (S) and threonine (T) and flanked by positively charged residues] is also present in zebrafish Slc16a2. It has a length of 33 amino acids and is flanked by histidine and arginine (amino acids 2 and 36, respectively, in the zebrafish Slc16a2 sequence). In mouse and rat Slc16a2, a second C-terminal PEST motif is present, and the human SLC16A2 sequence even contains three PEST domains (Fig. 1).

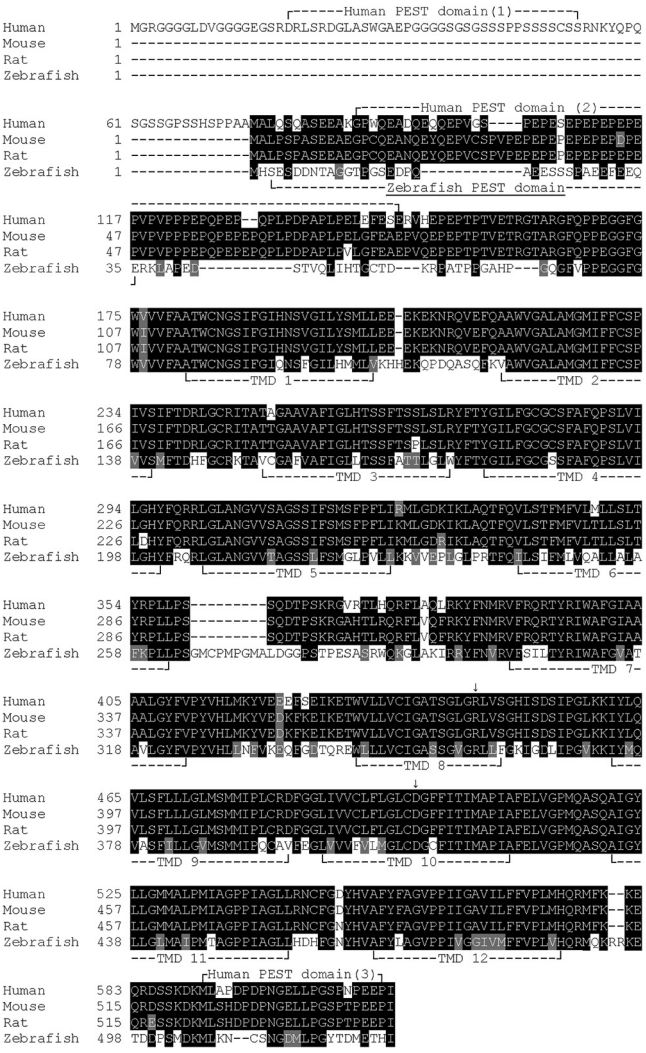

FIG. 1. Alignment of the zebrafish Slc16a2 protein (GenBank accession no. CBK52149) and its mammalian counterparts, which have been demonstrated to transport thyroid hormones: human SLC16A2 protein (GenBank accession no. NP_006508), mouse Slc16a2 protein (GenBank accession no. NP_033223), and rat Slc16a2 protein (GenBank accession no. NP_671749). Identical amino acids are *boxed in black*, conservative substitutions in *gray*. The predicted transmembrane α-helices in zebrafish are indicated by TMD1–TMD12. The human and zebrafish PEST domains are also indicated. Amino acids critically involved in thyroid hormone recognition and transport in humans (27), Arg^{445} and Asp^{498} are indicated by *arrowheads*.

Phylogeny of zebrafish Slc16a2 protein

A phylogenetic analysis of zebrafish Slc16a2 protein is presented in Fig. 2. Neighbor-joining analysis grouped the zebrafish sequence within the vertebrate SLC16A2/Slc16a2 clade. All vertebrate SLC16A2/Slc16a2 sequences cluster separately from SLC16A10/Slc16a10 sequences; teleostean, avian, and amphibian sequences form separate clusters again within Slc16a2.

Functional characterization of zebrafish Slc16a2 protein

Figure 3 shows the characterization of T_3 uptake by zebrafish Slc16a2. Because fish are ectotherms, we decided

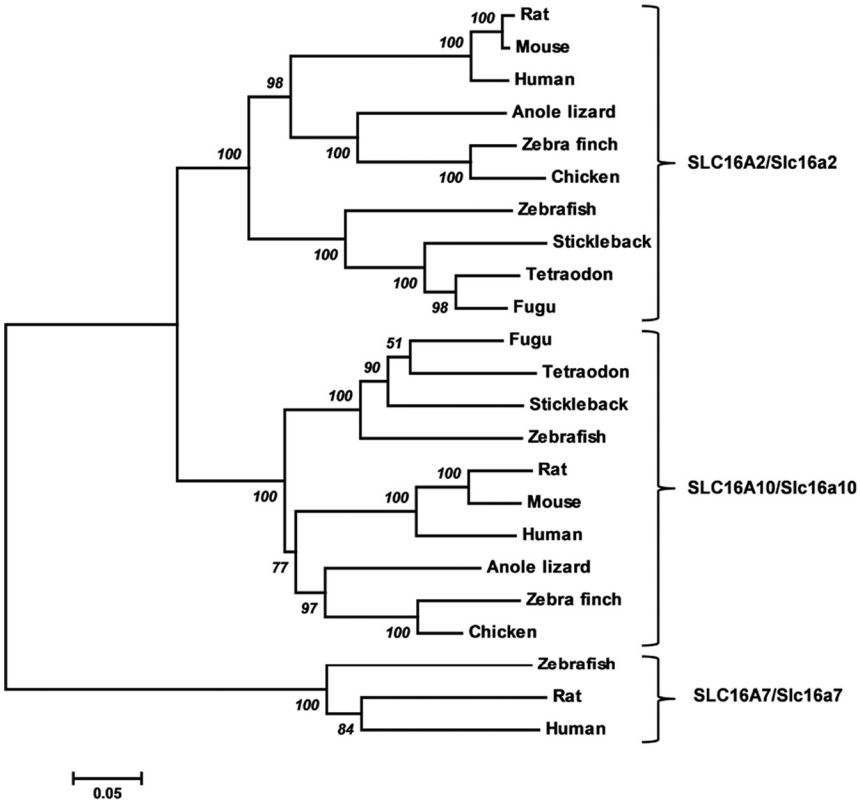

FIG. 2. Phylogenetic analysis of vertebrate SLC16A2/Slc16a2, SLC16A10/Slc16a10, and SLC16A7/Slc16a7 amino acid sequences using the neighbor-joining algorithm (other common names for these proteins are monocarboxylate transporter MCT8/Mct8, MCT10/Mct10, and MCT2/Mct2 respectively). Bootstrap test of phylogeny was performed with 1000 replications (*numbers at the branches* reflect the confidence level). SLC16A10/Slc16a10 and SLC16A7/Slc16a7 proteins were included as out-groups. GenBank accession numbers for the proteins included in the analysis are as follows: SLC16A2/Slc16a2: human, NP_006508; rat, NP_671749; mouse, NP_033223; anole lizard (*Anolis carolinensis*), ENSACAP00000002610; zebra finch (*Taeniopygia guttata*), XP_002195875; chicken (*Gallus gallus*), ENSGALP00000012547; tetraodon (*Tetraodon nigriviridis*), ENSTNIP00000019897; fugu (*Takifugu rubripes*), ENSTRUP00000038226; stickleback (*Gasterosteus aculeatus*), ENSGACP00000023345; zebrafish, CBK52149. SLC16A10/Slc16a10: human, NP_061063; rat, NP_620186; mouse, NP_001107804; anole lizard, ENSACAP00000011163; zebra finch, XP_002191589; chicken, ENSGALP00000024210; tetraodon, ENSTNIP00000006697; fugu, ENSTRUP00000044595; stickleback, ENSGACP00000008597; zebrafish, NP_001073497. SLC16A7/Slc16a7: human, NP_004722; rat, NP_058998; zebrafish, NP_001092889. Only full-length sequences were used for phylogenetic analysis.

to perform uptake assays at the rearing/body temperature of zebrafish (26 C). Figure 3A shows the time course of T_3 uptake by cells transfected with empty pcDNA 3.1 vector (control cells) and cells transfected with pcDNA 3.1 containing the zebrafish *Slc16a2* cds as an insert. The uptake of T_3 by cells expressing zebrafish *Slc16a2* was higher than that in control cells at all time points analyzed (from 3–300 min). Net T_3 uptake (difference between T_3 uptake in cells expressing zebrafish *Slc16a2* gene and control cells transfected with an empty plasmid) is linear up to 10 min and is adequately described by a first-order exponential equation, confirming the activity of a single transporter (Fig. 3B). The maximum rate, calculated from the slope of the tangent to the progress curve at time point 0, is 0.8 fmol T_3/min. The calculated first-order rate constant equals 0.06 min^{-1}. Figure 3C shows T_3-dependent saturable uptake of T_3 at an initial rate in control cells and cells transfected with zebrafish *Slc16a2*. Net initial uptake rates are well described by a single-site Michaelis-Menten function (calculated kinetic parameters were V_{max} = 582 fmol T_3/min, and K_m = 0.8 μM T_3). Data points and calculated V_{max} and K_m converge on a linear Eadie-Hofstee transformation, confirming the activity of a single active transporter (Fig. 3D). T_3 uptake by COS-1 cells expressing zebrafish *Slc16a2* and control cells increased when the incubation temperature during transport assays was increased from 26 C to 37 C (Fig. 3E). This increase in incubation temperature resulted in a 3- and 2-fold increase in net uptake of T_3 after 10 and 30 min incubation time, respectively. Replacement of Na^+ by choline$^+$ in the incubation medium did not significantly affect T_3 uptake (Fig. 3F).

At 26 C, no net uptake of T_4 (difference between T_4 uptake in cells expressing zebrafish *Slc16a2* gene and control cells) by zebrafish Slc16a2 could be measured (Fig. 4A). However, when cells were incubated at 37 C for 30 min, T_4 uptake was significantly higher in zebrafish *Slc16a2*-transfected cells compared with control cells (Fig. 4B). At 37 C, net T_4 uptake by zebrafish Slc16a2 was Na^+ independent (Fig. 4C).

Expression of *Slc16a2* transcripts in zebrafish tissues and during early development

Both type of tissue and time after fertilization were sources of variability for zebrafish *Slc16a2* gene expression in different tissues and during early development, respectively (one-way ANOVA, $P < 0.05$ and $P < 0.001$, respectively). The *Slc16a2* gene was ubiquitously expressed in all adult zebrafish tissues tested except in female gonads (Fig. 5). The highest expression levels were found in brain, followed by liver and kidney (brain > liver > kidney > gills, pancreas, pituitary, heart > gut). However, no significant differences were found between *Slc16a2* gene expression levels in these tissues. We also detected Slc16a2 mRNA in the following early developmental stages (Fig. 6): midblastula (3 hpf), gastrula (6 hpf), 6–10

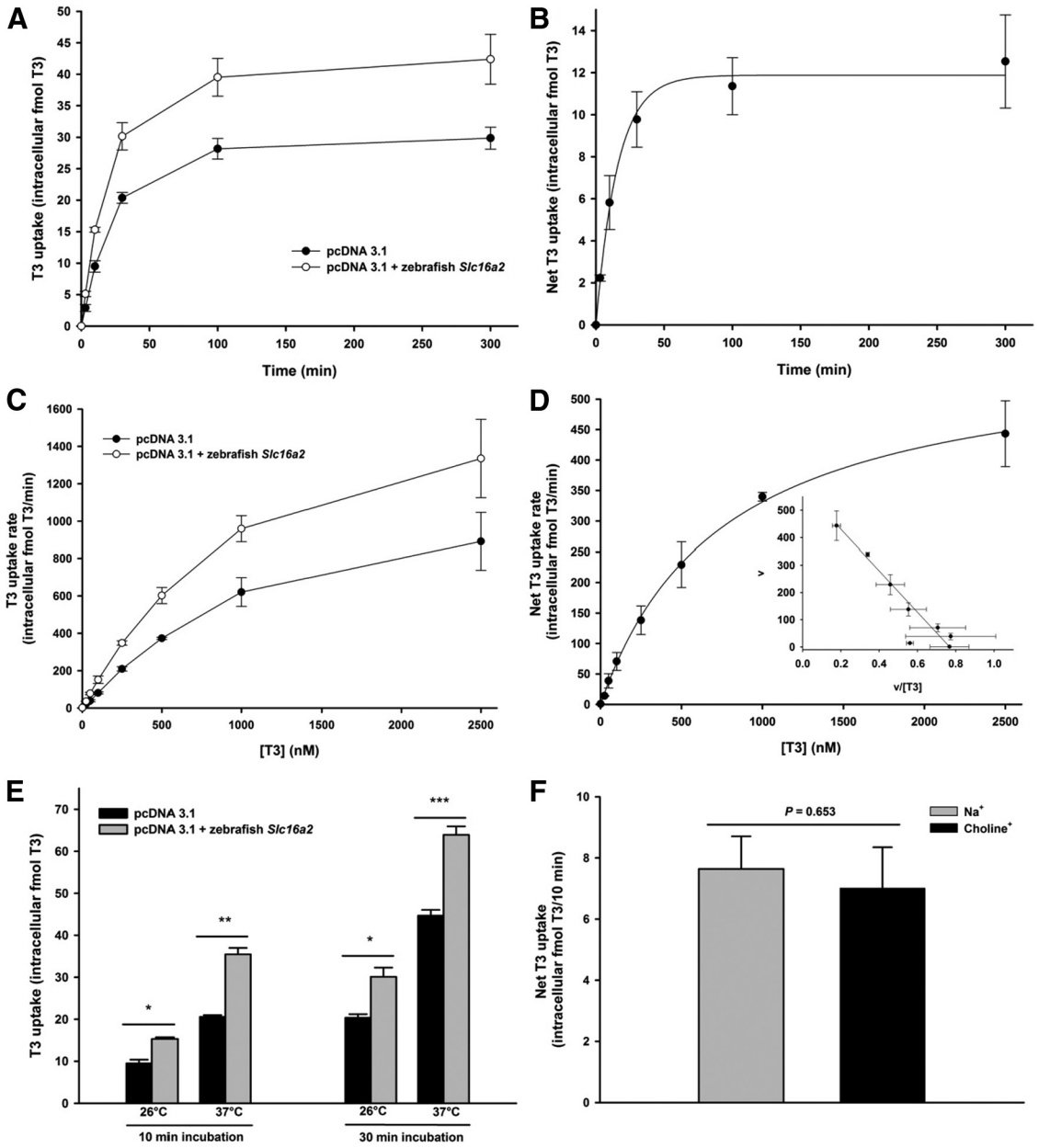

FIG. 3. Characterization of T_3 uptake in recombinant COS-1 cells seeded at a nominal density of 2×10^5 cells per well. Results are the mean ± SD of two to three independent experiments performed in duplicate. A, Time course of T_3 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing the cDNA coding for the zebrafish *Slc16a2* gene as an insert. B, Progress curve of net T_3 uptake in COS-1 cells (difference between T_3 uptake in cells expressing the zebrafish *Slc16a2* gene and control cells). The progress curve is well described by a first-order exponential equation: $f(t) = A_\infty \times (1 - e^{-kt})$. A_∞ is a limit at $t = \infty$, k is the first-order rate constant (minutes^{-1}), and t is time (minutes). Calculated kinetic parameters are $A_\infty = 12$ fmol T_3; $k = 0.06$ min^{-1}. The tangent to the curve at $t = 0$ gives the maximum net T_3 uptake rate, which is 0.8 fmol T_3/min. A and B, Uptake was measured at the rearing temperature of zebrafish (26 C) with 1 nM T_3 plus 2×10^5 cpm ^{125}I-labeled T_3 in DMEM/F12 medium plus 0.1% BSA. C, Substrate-dependent uptake of T_3 by COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish *Slc16a2* gene as an insert. D, Net T_3 uptake in COS-1 cells, measured at initial rate, as a function of substrate concentration. Data points are described by a single-site Michaelis-Menten function with $V_{max} = 582$ fmol T_3/min and $K_m = 0.8$ μM T_3. The *inset* shows an Eadie-Hofstee transformation of the data points and calculated kinetic parameters. C and D, Uptake was measured at initial rate (10 min incubation) and at the rearing temperature of zebrafish (26 C) with 1–2500 nM T_3 plus 2×10^5 cpm ^{125}I-labeled T_3 in DMEM/F12 medium plus 0.1% BSA. E, Temperature-dependent T_3 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish *Slc16a2* gene as an insert. Uptake was measured with 1 nM T_3 plus 2×10^5 cpm ^{125}I-labeled T_3 in DMEM/F12 medium plus 0.1% BSA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. F, Na^+ dependence of the net uptake of 1 nM T_3 plus 2×10^5 cpm ^{125}I-labeled T_3 in COS-1 cells expressing the zebrafish *Slc16a2* gene. Cells were incubated for 10 min at 26 C in a buffer containing NaCl or in a sodium-free medium containing an equimolar amount of choline.

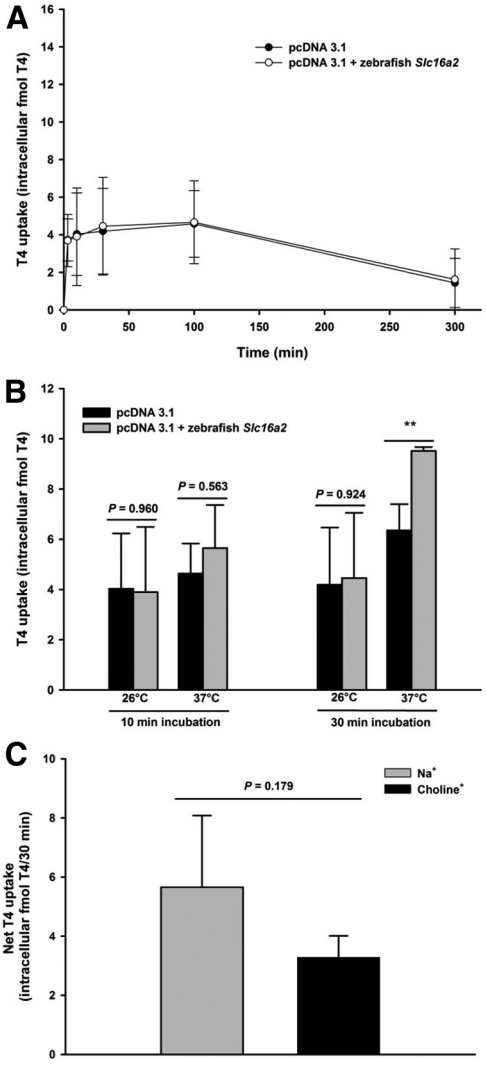

FIG. 4. Characterization of T_4 uptake in recombinant COS-1 cells seeded at a nominal density of 2×10^5 cells per well. Results are the mean ± SD of two to three independent experiments performed in duplicate. A, Time course of T_4 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish *Slc16a2* gene as an insert. Uptake was measured at the rearing temperature of zebrafish (26 C) with 1 nM T_4 plus 2×10^5 cpm ^{125}I-labeled T_4 in DMEM/F12 medium plus 0.1% BSA. B, Temperature-dependent T_4 uptake in COS-1 cells transfected with an empty pcDNA 3.1 vector (control cells) or a vector containing cDNA coding for the zebrafish *Slc16a2* gene as an insert. Uptake was measured with 1 nM T_4 plus 2×10^5 cpm ^{125}I-labeled T_4 in DMEM/F12 medium plus 0.1% BSA. **, $P < 0.01$. C, Na^+ dependence of the net uptake (difference between T_4 uptake in cells expressing zebrafish *Slc16a2* gene and control cells) of 1 nM T_4 plus 2×10^5 cpm ^{125}I-labeled T_4 in COS-1 cells expressing the zebrafish *Slc16a2* gene. Cells were incubated for 30 min at 37 C in a buffer containing NaCl or in a sodium-free medium containing an equimolar amount of choline.

somite segmentation (12 hpf), pharyngula (24 hpf), late pharyngula (48 hpf), and yolk sac larvae (72 hpf). The highest gene expression levels were found 48 hpf at which time zebrafish embryos had not hatched yet. At 48 hpf, *Slc16a2* gene expression levels were significantly higher than those at the other time points (Tukey's test, $P <$

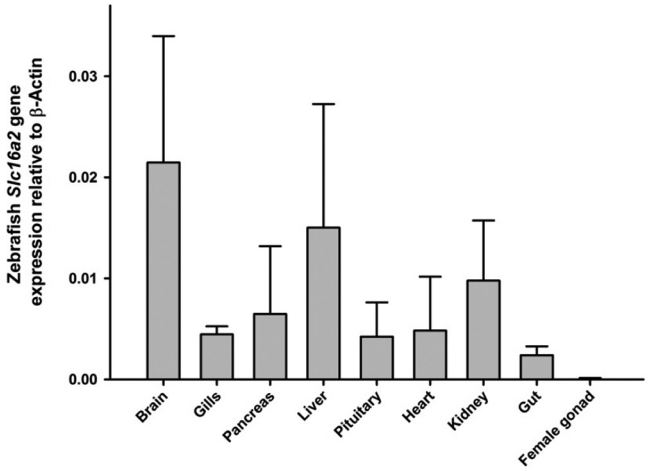

FIG. 5. Tissue distribution of zebrafish *Slc16a2* gene expression as determined using RT-qPCR. Primer oligonucleotide sequences are shown in Table 1. Dual internal standards (40S ribosomal protein S11 and β-actin) were incorporated in all measurements. Results were similar after standardization to either gene, and we therefore show only expression levels relative to β-actin. Constitutive expression of *Slc16a2* in zebrafish organs and tissues is corrected for primer efficiency and plotted as a ratio between target *vs.* reference gene. Data are presented as mean ± SD (n = 3 animals). The type of tissue is a source of variability for *Slc16a2* gene expression in different zebrafish tissues (one-way ANOVA, $P < 0.05$). Zebrafish *Slc16a2* gene expression levels in female gonads were significantly lower when compared with gene expression levels in other tissues (Tukey's test, $P < 0.05$).

0.001). Pairwise comparisons between *Slc16a2* gene expression levels at 3, 6, 12, 24, and 72 hpf were nonsignificant (Tukey's test, $P > 0.05$). Hatching occurred asynchronously in the 48- to 72-hpf time interval.

Discussion

Our studies demonstrate that the zebrafish Slc16a2 protein, an ortholog to the mammalian SLC16A2/Slc16a2 transporter, facilitates cellular thyroid hormone uptake in a temperature-dependent and Na^+-independent manner. This is the first characterization of a thyroid hormone membrane transporter in fish.

Zebrafish Slc16a2 shares not only a high sequence homology with human SLC16A2 and mouse and rat Slc16a2 but also 12 transmembrane α-helices with a high degree of conservation and an identical gene structure that consists of six exons. The only difference between the zebrafish and mammalian transporters is the presence of a single N-terminal PEST domain in zebrafish Slc16a2, whereas human, mouse, and rat sequences contain two to three PEST domains in the N and C termini. The PEST domain is thought to act as proteolytic signal, targeting the protein for rapid degradation (28). The low conservation of the zebrafish PEST sequence and the absence of a C-

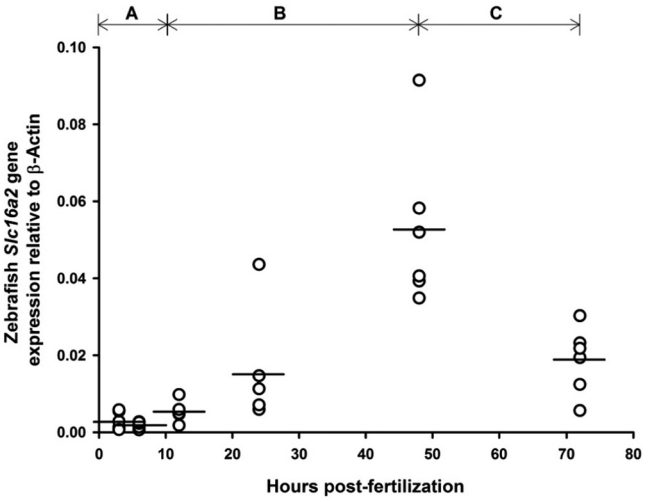

FIG. 6. Temporal pattern of zebrafish *Slc16a2* gene expression during early development, as determined using RT-qPCR. *Double-arrow segments* indicate three periods characterized by the following events: A, cell division and epiboly; B, organogenesis and morphogenesis of primary organ systems; C, hatching. Each *dot* represents individual determinations in embryos or larvae (n = 6). Means are indicated by *horizontal lines* within each group. Primer oligonucleotide sequences are shown in Table 1. Dual internal standards (40S ribosomal protein S11 and β-actin) were incorporated in all measurements. Results were similar after standardization to either gene, and we therefore show only expression levels relative to β-actin. Constitutive expression of *Slc16a2* in zebrafish embryos and larvae is corrected for primer efficiency and plotted as a ratio between target *vs.* reference gene. The time after fertilization is a source of variability for zebrafish *Slc16a2* gene expression during early development (one-way ANOVA, $P < 0.001$). *Post hoc* Tukey's test showed that *Slc16a2* gene expression levels at 48 hpf are significantly higher when compared with gene expression levels in the rest of the time points ($P < 0.001$).

terminal PEST motif in zebrafish Slc16a2 suggests a different regulation of protein turnover compared with its mammalian counterparts.

The high degree of conservation between transmembrane domains in zebrafish Slc16a2 and its mammalian orthologs as well as the presence in the zebrafish sequence of the two amino acids that are critical in substrate recognition in human SLC16A2, Arg^{445} and Asp^{498} (27), point to a conservation of function in the zebrafish Slc16a2 protein. When we measured thyroid hormone transport by zebrafish Slc16a2, we employed the ambient temperature of Slc16a2 in the living zebrafish, which was reared at 26 C. At this physiologically relevant temperature, cellular uptake of radiolabeled T_3 is a saturable process for which a K_m value of 0.8 μM was calculated. Both time course and kinetic analyses show that net T_3 uptake was performed by a single kinetic component. Compared with mammalian Slc16a2, the affinity of zebrafish Slc16a2 for T_3 *in vitro* is higher than that in mammals where K_m values for T_3 transport at 37 C are approximately 6- to 10-fold higher (27). Under normal physiological conditions, total plasma T_3 concentrations in zebrafish are approximately 1.5 nM (29), two to three orders of magnitude lower than the calculated K_m value for T_3 uptake in this study. It appears that zebrafish Slc16a2 *in vivo* is operating well below its limiting rate, and small elevations in plasma T_3 concentrations will result in a linear increase in uptake rate.

Thyroid hormone uptake by zebrafish Slc16a2 is temperature dependent, as it is for mammalian Slc16a2 (11). Zebrafish Slc16a2 transport activities are increased at the physiologically relevant temperature for mammals, 37 C, a temperature that fish do not tolerate. When measured at 37 C, zebrafish Slc16a2, as does human SLC16A2 (12), transports both T_3 and T_4, but only T_3 when the incubation temperature is that of the body temperature (*i.e.* 26 C). An intriguing but highly speculative notion would be that the acquired capacity for T_4 by the mammalian/endothermic Slc16a2 protein is an example of exaptation, *i.e.* a necessary byproduct of endothermy (30).

The *Slc16a2* gene is preferentially expressed in the adult zebrafish brain, gills, pancreas, liver, pituitary, heart, kidney, and gut. The tissue distribution is similar to that in rat (11), and it indicates these organs as important targets for thyroid hormone action. In the zebrafish brain, most likely, Slc16a2 regulates T_3 availability in a manner similar to that in mammals where SLC16A2 facilitates the transmembrane uptake of T_3 in neurons. This is a key process for neuronal activity because mammalian neurons do not possess a T_4-to-T_3 conversion capacity (2). In addition, zebrafish *Slc16a2* is also expressed during early development, where its gene expression reaches a maximum in the late pharyngula stage, at 48 hpf. In this stage, which is immediately before hatching, the morphogenesis of the main primary organ systems such as the thyroid gland (31) and the nervous, muscular, and circulatory systems (32) is completed. The temporal expression pattern indicates that Slc16a2 is involved in these major developmental changes, probably by facilitating an increase in intracellular T_3 availability, which regulates the expression of key morphogenetic target genes.

In conclusion, we have cloned the zebrafish *Slc16a2* gene (cds) and functionally characterized the zebrafish Slc16a2 protein as a thyroid hormone membrane transporter. Our findings are the first direct evidence for thyroid hormone membrane transporters in a piscine species. Zebrafish Slc16a2 is likely to play a key role in the local availability of T_3 during early development and in adult tissues.

Acknowledgments

We thank Marnix Gorissen, M.Sc., for technical assistance with zebrafish dissection, and Moniek van't Root and Iris Peters Rit for their help during procedures with embryos and larvae.

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F.J.A. is a recipient of a postdoctoral grant from the Ramón Areces Foundation (Madrid, Spain).

Disclosure Summary: E.d.V., T.J.V., G.F., and P.H.M.K. have nothing to declare.

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